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Sepsis syndrome stimulates proximal tubule cholesterol synthesis and suppresses the SR-B1 cholesterol transporter

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Sepsis syndrome stimulates proximal tubule cholesterol synthesis and suppresses the SR-B1 cholesterol transporter.

Background. Previous studies demonstrate that renal cortical/proximal tubule cholesterol accumulation is part of the renal "stress response." The present study was performed to help define underlying mechanisms, using experimental sepsis as a test model.

Methods. Male CD-1 mice and female low-density lipoprotein receptor (LDLR) knockout mice were injected with a heat-killed *Escherichia coli* suspension. Renal cortex and serum were obtained from these and control mice either 4, 6, or 18 hours later. Tissues samples were assayed for free cholesterol (FC), cholesteryl esters (CE), HMG CoA reductase (HMGCR) mRNA, and SR-B1 [the high-density lipoprotein (HDL) receptor/cholesterol transporter]. Statin effects on renal cortical HMGCR mRNA and FC/CE levels also were assessed. Finally, the impact of serum from septic versus normal mice on cultured proximal tubule (HK-2) cell cholesterol levels was assessed.

Results. Sepsis induced approximately 30% and 300 to 500% increases in renal FC and CE content, respectively. Cholesterol accumulation was not blunted in LDLR−/− mice versus their controls. Statin therapy also did not alter sepsis-induced renal FC/CE accumulation. However, statin treatment exerted no discernible intra-renal activity (for example, no rise in renal HMGCR mRNA), despite significant extra-renal activity (25% reduction in serum cholesterol; 400% increase in hepatic HMGCR mRNA). HK-2 cells exposed to septic serum sustained a 40% cholesterol increase, compared to cells exposed to control serum. This response was completely statin inhibited, proving that de novo synthesis was involved. Sepsis markedly suppressed renal levels of SR-B1 (an FC efflux protein). Renal HMGCR mRNA did not fall despite sepsis triggered cholesterol loading, indicating a failure of negative feedback activity.

Conclusions. Sepsis-induced renal cholesterol accumulation is not simply an intrinsic renal response, since it can be enhanced by circulating "stress factors" that drive HMGCR activity. Sepsis also down-regulates SR-B1. Thus, decreased cell FC efflux, coupled with increased synthesis, may synergistically induce the post-sepsis cholesterol overload state.

Key words: endotoxin, cholesteryl esters, free cholesterol, HMG CoA reductase, statins, LDL receptor.

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Previous work from this laboratory has demonstrated that renal cortical/proximal tubular cell cholesterol accumulation is an integral component of the kidney's response to tissue "stress" [1–9]. For example, within 24 to 48 hours following heterogeneous forms of renal injury (for example, renal ischemia, rhabdomyolysis, nephrotoxic serum nephritis, urinary tract obstruction, heat shock, dehydration, sepsis syndrome), 20 to 30% increments in free cholesterol (FC) levels result [1, 2, 4, 5]. A correlate of this process is a shuttling of FC into cholesteryl ester (CE) "storage" pools, causing the latter to rise as much as 10- to 20-fold over control tissue values [2, 4, 5]. While all of the metabolic consequences of these FC/CE increments remain to be defined, one documented result is increased proximal tubular cell resistance to superimposed ischemic or nephrotoxic attack, so-called acquired cytoresistance [10–18]. The mechanistic link between cytoresistance and cholesterol elevations has been established by experiments demonstrating that cholesterol reductions, induced by heterogeneous means, can both reverse acquired cytoresistance, and predispose otherwise normal cells to superimposed ischemic or toxic attack [1, 7, 8].

While cholesterol accumulation appears to be a ubiquitous response to acute renal injury, the mechanisms by which it occurs may vary, depending on the nature of the initiating insult. For example, in the case of heat shock and myoglobinuric renal injury, an increase in 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase (HMGCR) occurs, implying that increased renal cholesterol synthesis is involved [5]. That statin treatment prevents cholesterol accumulation in cultured HK-2 cells following Fe-mediated injury further supports this link [2]. In contrast, with sepsis syndrome no increase in renal cortical HMGCR protein results and it remains at control tissue levels [5]. This suggests that factors other than de novo cholesterol synthesis, for example, increased uptake of circulating cholesterol [such as, via the low-density lipoprotein (LDL) receptor], or decreased cellular cholesterol efflux (such as, via the scavenger receptor B1; SR-B1) may be involved [19–24].

Given that sepsis syndrome is such a frequent renal insult, the present study was undertaken to elucidate mechanisms by which renal cortical cholesterol accumulation results. To this end, we employed a previously described sepsis model (intraperitoneal injection of heat-killed *Escherichia coli*) for study because it induces renal cortical cholesterol overload without inducing overt histologic damage [5]. Hence, the results obtained should reflect tissue "stress" without the potentially confounding variables arising from tubular necrosis, cast formation, or other forms of renal histologic damage.

METHODS

Effect of sepsis on renal cortical cholesterol levels with and without statin therapy

As noted above, it has been previously established that experimental sepsis induces renal cortical cholesterol accumulation without an apparent increase in HMGCR protein [5]. However, it remains possible that increased activity of constitutively expressed HMGCR might still drive excess cholesterol synthesis. The following experiment was designed to test this possibility, using an HMGCR inhibitor as a pharmacologic probe. The hypothesis was as follows: if sepsis-induced cholesterol accumulation is HMGCR dependent, then statin administration theoretically should diminish this response. Toward this end, 20 CD-1 male mice (25 to 35 g; Charles River, Wilmington, MA, USA) were divided into two equal groups and received either (*1*) oral atorvastatin ("statin") containing diet [5]; or (*2*) a control diet. The atorvastatin diet consisted of normal mouse chow to which 1.5 mg atorvastatin (Pfizer/Warner Lambert; Ann Arbor, MI, USA) was added to each gram of chow. This was done by pulverizing the chow pellets, moisturizing it with water, adding atorvastatin, and then shaping it back into pellets and allowing them to dry. The control diet was treated in an identical fashion but without statin addition. This diet previously has been demonstrated not to affect weight gain, but it does induce an approximate 25% reduction in total serum cholesterol levels within three days [5]. After three days on these respective diets, the mice received intraperitoneal (IP) injections of 2 mL of a heat-killed *E coli* suspension ($\sim 1 \times 10^{10}$ organisms/mL normal saline), prepared as previously described [5]. In brief, colonies of *E. coli*, isolated from the urine of two patients with urosepsis, were expanded by culture in "Terrific" medium (Sigma, St. Louis, MO, USA). After three days in culture, at which time an obvious slurry was produced, the bacteria were pelleted by centrifugation, washed with saline, re-suspended in saline, and stored at –20°C. Prior to use, 2 mL aliquots were thawed and placed into a boiling water bath for 20 minutes, rendering the organisms non-viable but leaving endotoxin intact [25]. After cooling, the bacteria were ready for IP injection. Following the *E. coli* injections, the mice were returned to their former diets. Approximately 18 hours later, they were deeply anesthetized with pentobarbital (1 to 2 mg IP), the abdominal cavities were opened, and the mice were exanguinated from the inferior vena cava. One kidney per animal was removed, iced, and the cortex was subjected to chloroform:methanol extraction for subsequent analysis of cholesterol content [free cholesterol (FC) and cholesteryl esters (CE)] by gas chromatography (GC), as previously described [2]. The blood samples were saved and analyzed for blood urea nitrogen (BUN) and creatinine concentrations by autoanalyzer technology. As an additional surrogate marker of the severity of sepsis syndrome, plasma lactate dehydrogenase (LDH) levels, released from multiple tissues in response to injury, were assessed. To establish control values for the above assessments (that is, in the absence of sepsis), kidney and serum samples were obtained from six normal mice and subjected to the above renal cholesterol and plasma analyses.

HMGCR mRNA levels in the setting of sepsis

Twelve mice were injected with 2 mL of the heat-killed *E. coli* suspension. Either 4 or 18 hours later (N = 6 each), the mice were anesthetized with pentobarbital, the abdominal cavities were opened, a heparinized blood sample was obtained from the inferior vena cava (from the 18 hour mice), and then both kidneys were resected. The 18-hour plasma samples were saved for BUN and cholesterol analysis. The cortex of one kidney was used for RNA extraction and subsequent HMGCR mRNA and GADPH mRNA analysis by reverse transcriptase polymerase chain reaction (RT-PCR), as previously performed in this laboratory [9]. The other kidney was used to assess cholesterol levels. Twelve mice, injected with the *E. coli* carrier (saline), were treated in an identical fashion and used to collect control plasma and kidney samples at both the 4- and 18-hour time points. The mRNA results were expressed as HMGCR mRNA/GADPH mRNA ratios following densitometric analysis of band density/width [9].

Impact of statin therapy on HMGCR mRNA expression in normal mice

The following experiment was undertaken to ascertain whether statin therapy increases renal HMGCR mRNA expression in normal mice, as would be expected if the drug effectively inhibits renal HMGCR enzyme activity (that is, a compensatory increase in HMGCR mRNA should result). Six mice were fed either the atorvastatin or the control diet (N = 3 each) for three days. The mice were then anesthetized with pentobarbital, a plasma sample was obtained for cholesterol analysis, and then the kidneys, and a piece of liver, were removed. Both hepatic and renal tissues (1 kidney per animal) were

extracted for RNA and subsequently analyzed for HMGCR mRNA, as noted above. The second kidney was used for FC and CE analysis.

Cultured HK-2 proximal tubular cell cholesterol homeostasis

Impact of sepsis serum. The following experiment was conducted to assess whether a septic milieu, mimicked in vitro, can induce alterations in proximal tubular cell cholesterol homeostasis. To this end, six mice were injected with the *E. coli* preparation. Six additional mice received the *E. coli* vehicle (2 mL sterile saline). Approximately four hours later, the mice were anesthetized with pentobarbital and ~0.5 mL blood samples per mouse were obtained from the inferior vena cava. The sera were separated and subjected to complement inactivation (heating at 56°C ×30 min). Pools of serum from both septic and control mice were prepared, and then aliquots were added to subconfluent proximal tubule (HK-2) cells grown in T25 flasks ×3 days ($N = 6$ per group). Prior to the serum addition, the cells were maintained in keratinocyte serum-free medium (K-SFM) as previously described [26]. The serum samples were added to achieve a final concentration of approximately 13.5%. The cells were incubated for 18 hours and then the media samples were removed and saved. The flasks were placed on ice, rinsed twice with Hanks balanced salt solution (HBSS), and then the cells were collected with a scraper. The cells were pelleted by centrifugation, and the lipids were extracted in chloroform:methanol [5]. Total cholesterol content was measured with a commercially available enzymatic assay kit (Sigma), as previously used and validated in this laboratory [1]. (Note: this enzymatic assay, rather than GC analysis, was used because of the relatively small sample sizes obtained from T25 flasks. T25 flasks, rather than larger flasks, were used because of the small quantities of serum that could be obtained from each mouse). Total cholesterol values were expressed as nmoles cholesterol/μmol phospholipid phosphate [1]. Additionally, the cholesterol content in the media was determined to calculate the starting whole serum cholesterol level.

Impact of statin therapy on sepsis serum-mediated cellular cholesterol increases. The following experiment was performed to assess whether HMGCR inhibition would block increases in cellular cholesterol that were evoked by the addition of sepsis serum (**Results** section). To this end, the experiment described earlier in this study was repeated exactly, with the exception that 10 μmol/L mevastatin was added to each of the flasks at the same time as the serum addition. Of note, it was determined previously that this mevastatin concentration effectively inhibits HK-2 cell HMGCR [5].

Direct impact of bacteria exposure on HK-2 cell cholesterol levels. The following experiment was undertaken to ascertain whether direct exposure to *E. coli*/endotoxin directly alters tubular cell cholesterol accumulation. To this end, six T25 flasks of HK-2 cells were divided into two groups of three: (*1*) exposure to the *E. coli* preparation (0.33 mL per 5 mL of culture media that contained 13.5% heat-inactivated normal mouse serum ($N = 3$; Gibco, Grand Island, NY, USA); or (*2*) addition of 0.33 mL of normal saline, instead of the *E. coli* ($N = 3$). After the 18 hour incubations, total cell cholesterol was determined, as noted earlier in this article.

SR-B1 expression following the induction of sepsis

Scavenger receptor B1 (SR-B1) can significantly impact cellular FC and CE levels in cells, both by increasing the CE influx and by driving the FC efflux by facilitation of its aqueous diffusion through the plasma membrane to circulating HDL [21–23]. Hence, the goal of this experiment was to determine whether acute sepsis syndrome, with its attendant renal cholesterol increments, alters SR-B1 expression. To this end, 16 mice were subjected to the sepsis protocol, and after either four ($N = 4$), six ($N = 4$), or 18 ($N = 6$) hours the mice were anesthetized with pentobarbital and the kidneys were removed. Simultaneously obtained kidneys from mice subjected to saline carrier, rather than *E. coli*, injection were harvested also ($N = 4$ at each of the three time points). The kidneys were iced, the cortices dissected, and prepared for Western blot analysis, as previously described [5]. Seven micrograms of protein extract were electrophoresed through a 12% Bis-Tris acrylamide gel (Invitrogen, Carlsbad, CA, USA) and then probed with rabbit polyclonal anti-SR-B1 antibody (catalog number NB-400-104; Novus Biologicals, Littleton, CO, USA). Donkey anti-rabbit IgG (peroxidase-linked, species specific whole antibody; Amersham Pharmacia Biotech, Uppsala, Sweden) was used as the secondary antibody. Detection was by enhanced chemiluminescence (ECL), as previously described [5]. To confirm the specificity of the Western analysis, gels were run in the same fashion, except for the omission of the primary anti-SR-B1 antibody. Equal protein loading/transfer was confirmed by India ink staining of the gels. Statistical comparisons of SR-B1 expression was assessed by subjecting the SR-B1 band to densitometric analysis, followed by separate unpaired Student *t* tests for the four, six, and 18 hour results.

To determine whether changes in SR-B1 in the above experiments are unique to sepsis, its expression in a second model of cell injury-induced cholesterol overload was studied: the glycerol-induced ARF model [4, 18]. To this end, six mice were injected with 10 mL/kg of 50% glycerol (equally divided IM doses into each upper hind limb). Eighteen hours later they were anesthetized; the kidneys were removed, and renal cortical protein extracts probed for SR-B1, as noted earlier. The results

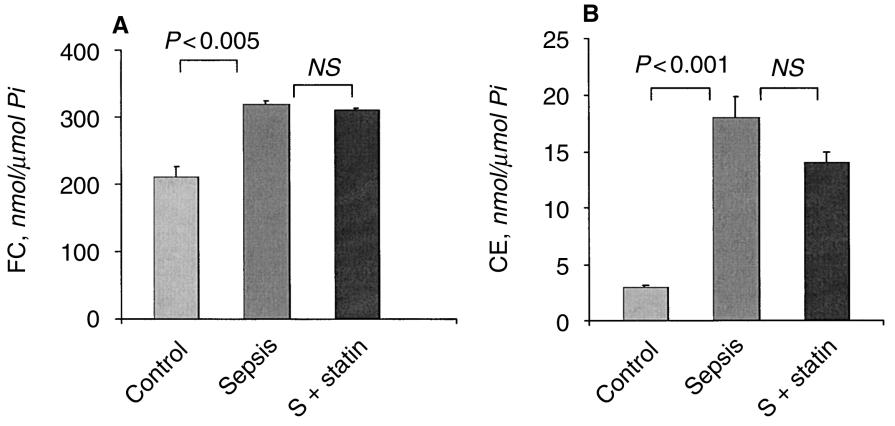

Fig. 1. Renal cortical free cholesterol (FC) and cholesteryl ester (CE) levels in control mice and in mice 18 hours following *E. coli* injection (sepsis), or sepsis (S) + concomitant statin treatment (S + statin). Sepsis caused a dramatic increase in both FC and CE levels, and this result was not significantly blunted by concomitant statin treatment.

were compared to those that had been obtained simultaneously using cortical tissues from five normal mice.

LDL receptor knockout mouse experiments

To ascertain the impact of the LDL receptor (LDLR) on renal cholesterol levels in the presence and absence of sepsis, eight female LDL knockout mice (B6.129S7-Ldlr^{tm1Her}) of three to five weeks of age were obtained from Jackson Laboratory (Bar Harbor, ME, USA) [20]. Eight C57BL6J mice (Jackson Laboratory) of the same age were used as controls. The mice were maintained on a regular rodent diet. The day of experimentation, four knockout mice and four control mice were injected with 2 mL of the *E. coli* suspension. Eighteen hours later, the mice were anesthetized with pentobarbital, and then plasma was obtained from the inferior vena cava for BUN and cholesterol analysis. The kidneys were removed and processed for renal cortical cholesterol (FC, CE) analysis by GC.

To assess baseline parameters, the remaining four knockout mice and four control mice were anesthetized, the abdominal cavities opened, and plasma and renal cortical tissues were collected and analyzed, as noted above.

Impact of sepsis on isolated proximal tubule injury responses and cholesterol levels

The following experiment had two aims: (*1*) test whether cholesterol overload following sepsis is associated with the cytoresistant state; and (*2*) ascertain whether the renal cortical cholesterol elevations were expressed at the proximal tubular cell level. Five mice were injected with 2 mL of heat-killed *E. coli*. Five simultaneously treated mice received the *E. coli* vehicle. Eighteen hours later, the mice were anesthetized with pentobarbital and the kidneys were removed, and iced. The cortices were dissected and then used to isolate proximal tubule segments, as previously described in detail [16–18]. Tubules from one control mouse and one post-sepsis mouse were studied simultaneously. After completing a 15-minute re-warming period (from 4°C to 37°C), each tubule preparation was suspended in an experimentation buffer [16] to a concentration of approximately 2 mg tubule protein/mL and then divided into three equal aliquots and incubated as follows: (*1*) control incubation ×30 minutes (95% O_2/5% CO_2); (*2*) incubation with an Fe-mediated oxidant challenge [25 μmol/L ferrous ammonium sulfate, complexed with hydroxyquinoline (FeHQ)] a siderophore, allowing Fe to gain intracellular access [2]; and (*3*) 12.5 minutes of hypoxic incubation (95% N_2/5% CO_2) followed by 17.5 minutes of re-oxygenation (95% O_2/5% CO_2) [16]. At the completion of the 30 minute incubations, cellular injury in the control and post-sepsis tubules were contrasted by % LDH release [16]. Additionally, the control incubated tubules from the normal and post-sepsis mice were subjected to lipid extraction in chloroform:methanol and then analyzed for FC and CE content.

Calculations and statistics

All values are presented as means ± 1 SEM. Cholesterol levels were expressed as nmol/μmol phospholipid phosphate (Pi). Results between groups were contrasted by either the paired or unpaired Student *t* test. If multiple comparisons were made with any set of data, the Bonferroni correction was applied. Statistical significance was judged by a P value of <0.05.

RESULTS

Effect of sepsis on renal cortical cholesterol levels with/without statin therapy

As shown in Figure 1, both free cholesterol (FC) and cholesteryl esters (CEs) were significantly elevated by 18 hours post-induction of sepsis, compared to controls. The use of statin caused no significant decrease in these values. Figure 2 shows that the induction of sepsis also induced a "pre-renal" like state, causing marked elevations in BUN (Fig. 2A), but not plasma creatinine concentrations (Fig. 2B). Supporting a hemodynamic, rather

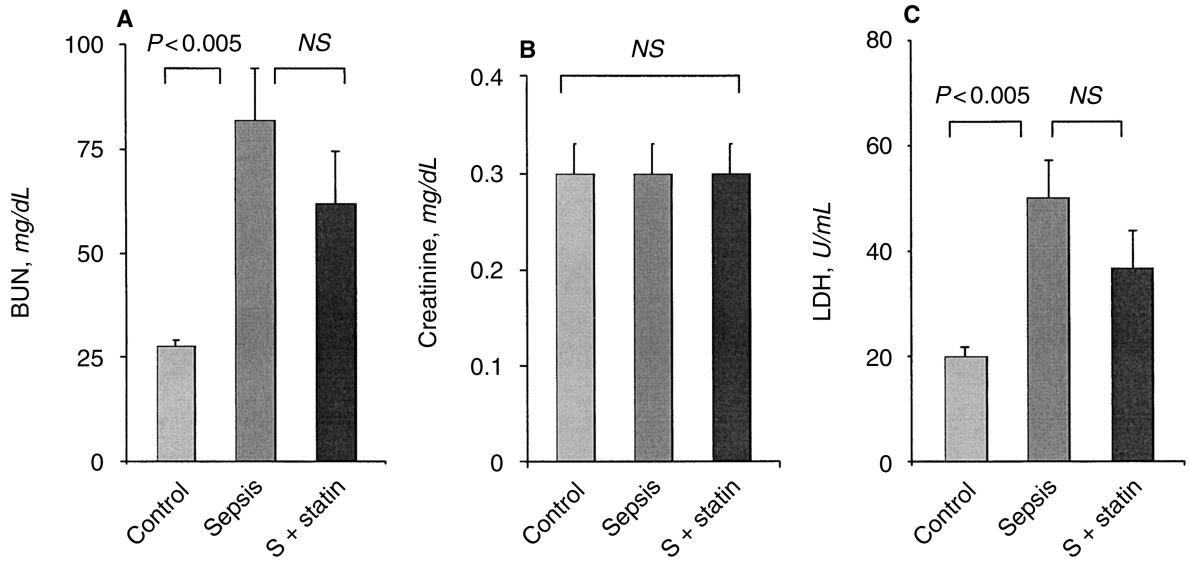

Fig. 2. Effect of *E. coli* injection on blood urea nitrogen (BUN), creatinine, and plasma lactate dehydrogenase (LDH) levels in the presence or absence of statins. *E. coli*-induced sepsis (S) caused a significant increase in BUN and LDH, but not in plasma creatinine levels. These changes were not significantly blunted by concomitant statin treatment (S + statin).

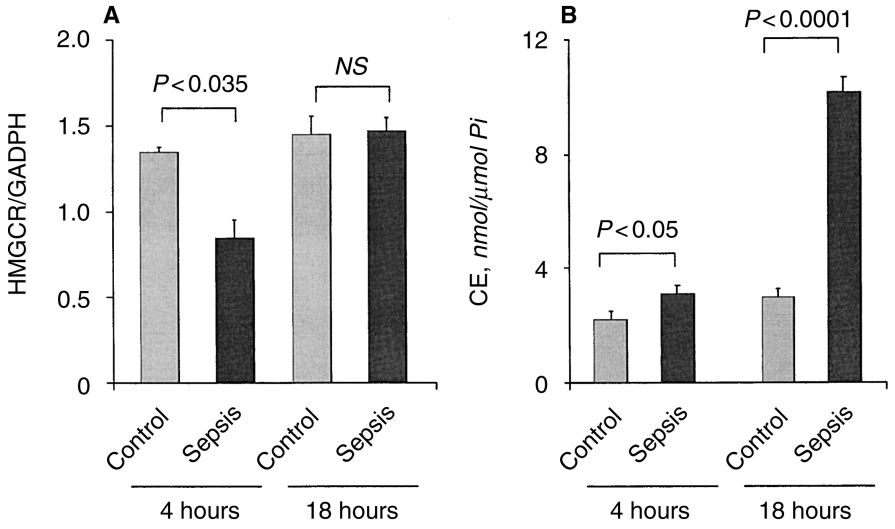

Fig. 3. HMGCR mRNA, factored by GADPH mRNA, following induction of sepsis syndrome. HMGCR mRNA levels were significantly depressed in renal cortex 4 hours following *E. coli* injection, compared to paired controls. However, by 18 hours post-sepsis, HMGCR mRNA had returned to completely normal values. This was despite marked tissue cholesterol overload at this time, indicating a lack of normal physiologic HMGCR mRNA suppression.

than a structural, basis for this ARF are previous observations of complete preservation of normal renal histology in this sepsis model, despite the azotemic state [5]. The degree of azotemia was slightly lower in the statin treated animals, but this difference did not achieve statistical significance. Sepsis also induced substantial, but highly variable, increases in the plasma LDH values (Fig. 2C). As with the BUNs, the LDH values were modestly, but not significantly, lower in the statin group. The LDH values positively correlated with the prevailing BUNs for both the control sepsis and statin treated groups ($r = 0.67$, $P < 0.05$; $r = 0.83$, $P < 0.01$; control and statin treated groups, respectively; data not shown). Thus, in summary, statin therapy appeared to slightly decrease the severity of sepsis, but given the highly variable nature of the BUN/LDH increments, these changes did not achieve statistical significance.

HMGCR mRNA analysis following sepsis

Renal HMGCR mRNA levels were suppressed by 33% after four hours of sepsis ($P < 0.035$), as depicted in Figure 3A. At this time, FC levels did not significantly differ between the control and sepsis group (168 ± 3 and 178 ± 3 nmol/μmol phospholipid phosphate, respectively). However, a slight increase in CE levels was apparent (Fig. 3B).

By 18 hours post-sepsis, HMGCR mRNA values were identical for the control and sepsis groups (Fig. 3A). At

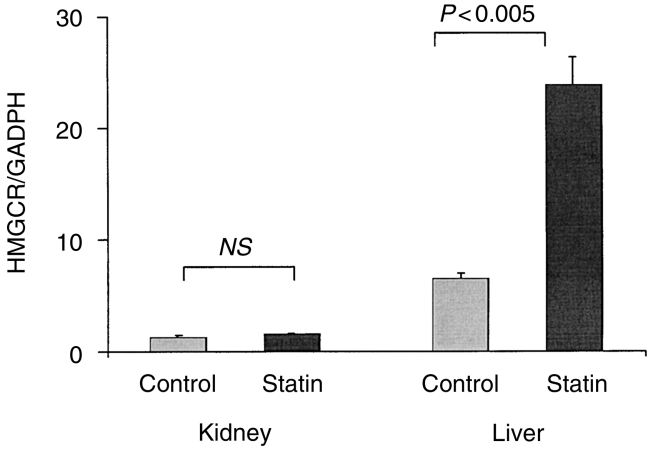

Fig. 4. HMGCR mRNA levels in kidney and liver following three days of oral statin therapy. Statin treatment caused no significant change in renal cortical HMGCR mRNA levels, compared to control kidney values. However, statin treatment induced a dramatic increase in hepatic HMGCR mRNA values. Hence, these results suggest that oral statin treatment has hepatic, but not significant renal bioactivity.

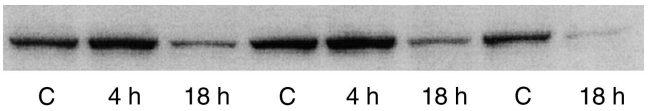

Fig. 5. Scavenger receptor-B1 Western blots of control (C) renal cortical tissues, and of renal tissues harvested either 4 or 18 hours post-induction of sepsis (S). As is readily apparent, a profound suppression of SR-B1 is apparent in the 18-hour post-sepsis samples. The 4 hour samples did not significantly differ.

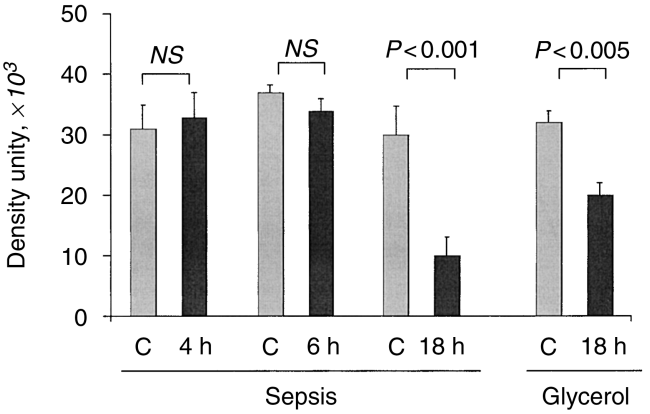

Fig. 6. Quantitation of SR-B1 in renal cortex by densitometric analysis of Western blots. SR-B1 expression did not significantly differ from normal at either 4 or 6 hours post-sepsis induction. However, by 18 hours, a marked reduction was apparent. Similarly, at 18 hours post-glycerol-induced rhabdomyolysis, SR-B1 levels were also significantly suppressed.

this time point, the sepsis group manifested the characteristic increases in FC (controls, 170 ± 5; sepsis, 195 ± 3; $P < 0.0001$) and CE levels (Fig. 3B). At the 18-hour time point, the sepsis model once again induced a significant increase in BUN concentrations (control BUNs, 29 ± 1; sepsis, 87 ± 14 mg/dL; $P < 0.002$). Sepsis also caused a slight increase in serum cholesterol concentrations (control, 127 ± 4; sepsis, 149 ± 8 mg/dL; $P < 0.04$).

Impact of statin therapy on renal and hepatic HMGCR mRNA expression

As shown in Figure 4, three days of statin therapy had no discernible effect on renal cortical HMGCR mRNA levels. In contrast, liver samples from these same animals demonstrated that statin therapy caused an approximate fourfold increase in HMGCR mRNA (a "positive control" for the negative kidney results). [Of note, HMGCR mRNA levels were markedly higher in liver vs. kidney at baseline, consistent with the fact that the liver is the dominant site of cholesterol synthesis.]

Statin effects on plasma and renal cortical cholesterol levels in normal mice

Serum cholesterols were reduced by approximately 25% after three days of statin therapy (134 ± 12 vs.102 ± 11 mg/dL; $P < 0.01$). Statin therapy also caused a modest decrease in renal cortical CE levels (3.8 ± 0.3 vs. 3.1 ± 0.4 nmol/μmol Pi; $P < 0.015$; controls vs. statin, respectively). In contrast, statin treatment had absolutely no effect on renal cortical FC levels (211 ± 15 vs. 211 ± 17; control vs. statin, respectively). Thus, these data are consistent with the earlier HMGCR mRNA data: they each imply that statin therapy had a minimal, if any, direct effect on renal HMGCR activity. [Indeed, the decrease in renal CE levels could simply have reflected lower CE uptake from the serum because of statin-induced reductions in cholesteryl ester levels.]

SR-B1 expression

Western blot analysis of post sepsis kidneys demonstrated a single band at approximately 55 kD (Fig. 5). This band was completely absent when the anti-SR-B1 primary antibody was deleted from the reaction. There was no significant change in SR-B1 expression at either four or six hours post-induction of the sepsis syndrome (Fig. 6). However, by 18 hours post-sepsis, an approximate 70% reduction in SR-B1 was apparent ($P < 0.001$). The corresponding cholesterol analysis performed on the contralateral kidney from that used for Western blotting demonstrated that at four hours post-induction of sepsis, no change in either FC (173 ± 2 vs. 175 ± 3) or in CE levels (3.6 ± 0.5 vs. 2.6 ± 0.3) existed (control vs. sepsis, respectively). By six hours, a modest increase in FC (179 ± 3 vs. 190 ± 2; $P < 0.04$), but not in CE (4.5 ± 0.2 vs. 4.7 ± 0.1; NS) was observed. However, by 18 hours post-sepsis, both FC (176 ± 2 vs. 196 ± 3; $P < 0.0001$) and CE values (3.9 ± 0.2 vs. 10.5 ± 1.0; $P < 0.0001$) were elevated in the sepsis animals. The BUNs

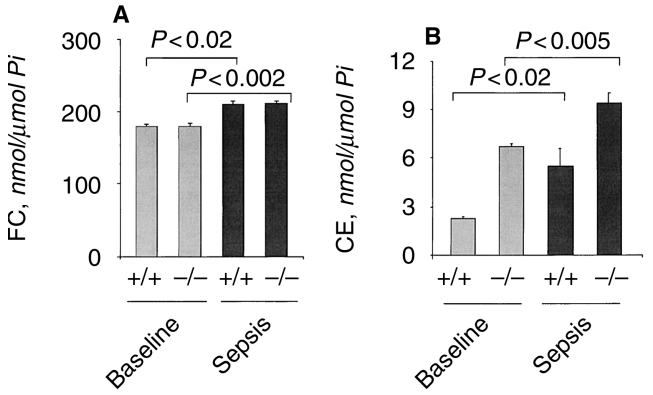

Fig. 7. Free cholesterol (FC) and cholesteryl ester (CE) levels in renal cortex both under baseline conditions and 18 hours post-sepsis in control (+/+) and LDL receptor knockout (−/−) mice. Baseline FC levels were identical for both groups of mice, and each group manifested essentially identical FC increases 18 hours post-induction of sepsis. In contrast, the knockout mice had significantly higher CE levels at baseline compared to the controls. However, each group developed comparable (and significant) CE increases in response to sepsis.

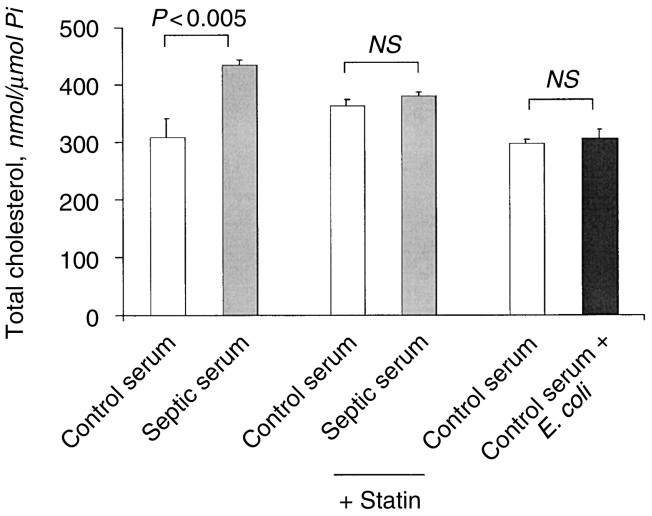

Fig. 8. Total cholesterol levels in HK-2 cells following incubations with either control or sepsis serum. Incubating cells with serum harvested from septic mice caused a significant increase in cholesterol levels (left panels). As shown in the middle panels, statin treatment (of normal or septic serum) completely abolished this response. Incubating cells with *E. coli* did not alter cholesterol levels (indicating that the aforementioned cholesterol increase was not directly due to bacterial products).

for these animals were as follows: sepsis, 87 ± 16; controls, 31 ± 1 mg/dL ($P < 0.01$).

Western blotting of 18 hours post-glycerol-induced rhabdomyolysis kidneys demonstrated an approximate 40% reduction in SR-B1 expression, compared to controls (Fig. 6). Hence, this reproduced the findings with the post-sepsis kidneys. The BUN values for the post-glycerol and control mice used for this experiment were 173 ± 21 and 30 ± 2 mg/dL, respectively ($P < 0.01$).

LDL receptor knockout mouse experiments

Baseline conditions. Baseline renal cortical FC and CE levels in the LDLR knockout (−/−) mice and control mice (+/+) are presented in Figure 7A. Renal cortical FC values were identical for the two groups. However, as shown in Figure 7B, the knockout group did have a higher CE content (likely reflecting compensatory increases in SR-B1 mediated CE uptake, as previously noted in this model [24]). As expected [20], serum cholesterol values were significantly higher in the knockout mice (212 ± 6 mg/dL) compared to the controls (68 ± 4; $P < 0.001$). This confirms successful induction of the knockout state (which leads to hypercholesterolemia due to loss of the LDL receptor). Renal function for the control and knockout mice appeared the same, at least as assessed by BUN concentrations (28 ± 2 and 29 ± 2 mg/dL, respectively).

Post-sepsis conditions. Both groups of mice responded to sepsis with statistically significant increases in renal cortical FC and CE concentrations (Fig. 7). In the case of FC, the post-sepsis increases were essentially identical for the two groups (Fig. 7A). The post-sepsis CE levels were higher in the knockout mice versus the controls. However, this difference simply reflected the baseline difference, and not a different degree of CE accumulation in response to sepsis. Indeed, if the baseline difference is subtracted from the post-sepsis difference, essentially identical CE elevations were apparent for the two groups. Thus, loss of the LDL receptor did not alter the sepsis-induced renal cholesterol accumulation. Interestingly, the decline in renal function post-sepsis was worse in the knockout versus the control mice (BUN 92 ± 6 mg/dL vs. 49 ± 12 mg/dL respectively; $P < 0.035$).

HK-2 cell cholesterol levels following incubation with "septic serum"

Incubation of HK-2 cells with serum harvested four hours after the induction of sepsis (septic serum, SS) demonstrated an approximate 40% increase in total cholesterol levels, compared to cells subjected to control serum (CS) incubation (Fig. 8, left). When this experiment was repeated in the presence of mevastatin ("statin"), the septic serum was no longer able to increase HK-2 cell cholesterol levels (Fig. 8, middle). At the time of serum harvesting, no difference in serum cholesterol existed between these two groups (128 ± 3 vs. 122 ± 5 mg/dL; sepsis vs. controls). Unlike the results with septic serum, addition of *E. coli* suspension did not alter the cell cholesterol levels (Fig. 8, right).

Isolated proximal tubule segment (PTS) experiments

Isolated tubules harvested from the sepsis mice had statistically significant increases in both FC and CE content, compared to control tubules (FC, 225 ± 3 vs. 181 ±

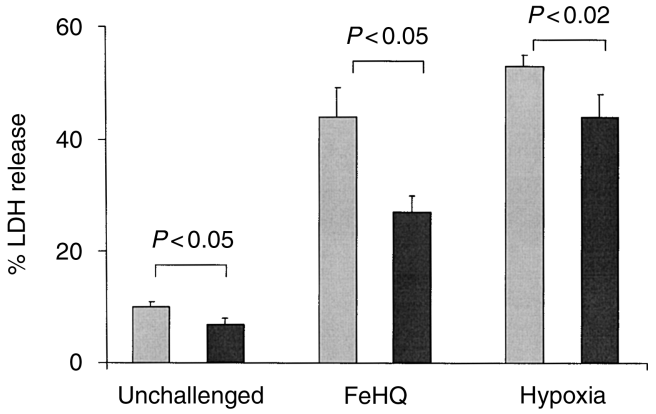

Fig. 9. Responses of isolated proximal tubules harvested from control (□) and post-sepsis (■) mice to control oxygenated incubation, incubation with an oxidant challenge (FeHQ), or hypoxic/re-oxygenation (hypoxia) injury. Under each of these circumstances, the post-septic tubules manifested less cell injury, as denoted by a significant decrease in % LDH release.

3; $P < 0.002$, respectively; CE, 6.7 ± 0.8 vs. 1.0 ± 0.1; $P < 0.002$, respectively). This confirmed that the post-sepsis FC/CE increases observed in whole renal cortex reflected, at least in part, proximal tubular cell events. As shown in Figure 9, PTS harvested from the post-sepsis mice showed significantly less cell injury in response to either the FeHQ-mediated oxidant challenge or hypoxia-reoxygenation injury. Even under control incubation conditions, the post-sepsis tubules manifested slightly greater cell viability than did the control tubules (Fig. 9). Thus, these proximal tubules exhibited cholesterol elevations and partial resistance to injury, each characteristic of the stress-induced cytoresistant state [16–18].

DISCUSSION

Cholesterol accumulation following renal injury indicates marked dysregulation of a series of highly regulated, interactive processes that normally maintain tissue cholesterol content within a narrow physiologic range. These processes include: (*1*) cholesterol uptake from plasma via LDL and non-LDL dependent pathways; (*2*) intrarenal cholesterol synthesis, with HMGCR being the critically regulated step; and (*3*) cellular cholesterol efflux [19]. To date, our studies of post-renal injury cholesterol accumulation have elucidated only one abnormality: an up-regulation of HMGCR protein that appears to drive excess cholesterol synthesis. This conclusion has been based on the findings of increased HMGCR protein in post-injured renal tissues by Western blotting [5], and that statin treatment blocks cholesterol accumulation in post-injured cultured HK-2 cells [2]. The mechanism for the increase in post-injury HMGCR protein remains incompletely defined. However, recent studies from this laboratory strongly suggest that both pre- and post-translational events can be involved [9].

Sepsis syndrome is one form of renal stress in which cholesterol overload develops despite apparently normal HMGCR protein expression [5]. This raises the question as to whether constitutively expressed HMGCR could be stimulated to drive cholesterol accumulation [26–30]. Any increase in tissue cholesterol levels resulting from de novo synthesis would then be magnified if either exaggerated cell cholesterol uptake, or reduced cell efflux, co-existed. The present study was designed to address such possibilities.

Seemingly the best way to ascertain the contribution of HMGCR to sepsis-induced cholesterol accumulation would be to treat animals with a statin and then determine whether this abrogates the cholesterol overload state. As depicted in Figure 1, oral statin therapy failed to significantly blunt post-sepsis cholesterol increments. The bioactivity of the statin treatment protocol is indicated by the fact that it successfully lowered serum cholesterol levels. Therefore, if taken at face value, these results suggest that HMGCR activity is not involved in sepsis-mediated renal cholesterol accumulation.

A potential caveat to the above conclusion is that statins undergo enterohepatic circulation, resulting in their being concentrated in liver. This raises the possibility that minimal renal accumulation, and hence, bioactivity, results. If this is so, then the failure of statin treatment to prevent sepsis-induced cholesterol accumulation could not be taken as evidence against an HMGCR-dependent mechanism. To address this possibility, the relative impact of statin therapy on renal HMGCR mRNA expression was assessed. The rationale for this experiment is that tissue HMGCR inhibition should cause a prompt rise in message expression, thereby proving that renal-specific bioactivity exists. As expected, statin therapy induced a fourfold rise in hepatic HMGCR mRNA, confirming activity in liver. However, no simultaneous change in renal HMGCR mRNA resulted. Statin therapy also failed to induce any significant change in renal free cholesterol levels. Given these observations, it would appear that minimal, if any, statin bioactivity existed within the kidney. Hence, the negative results with statin therapy, as shown in Figure 1, cannot be taken as evidence against HMGCR-mediated renal cholesterol accumulation. These findings have implications far beyond the issue of mechanisms of stress-induced renal cholesterol accumulation. For example, it has been suggested that statins may slow the progression of chronic renal disease [31, 32]. However, if this is the case, the present results imply that such an action might simply stem from changes in circulating lipids and/or cytokine expression, rather than a direct intra-renal statin effect. However, at this time, we cannot exclude the possibility that statins might impact HMGCR in specific cortical tissue compartments (for example,

endothelial or glomerular cells), since changes in their mRNA expression could have been masked by whole cortex analysis.

An intriguing question is whether 'normal' levels of HMGCR protein in the setting of sepsis actually reflect dysregulation of the HMGCR axis. Under physiologic conditions, cholesterol overload would be expected to down-regulate HMGCR expression due to suppression of stimulatory transcription factor expression (most notably, SREBP-2) [33–36]. This should then cause a sharp decline in HMGCR mRNA, followed by reduced HMGCR protein. Thus, our previous observation that sepsis-induced renal cholesterol accumulation is expressed in the presence of "normal" HMGCR protein levels [5] suggests that normal physiologic feedback inhibition did not exist. To explore this theme further, HMGCR mRNA levels have now been assessed. At four hours post-sepsis, HMGCR mRNA levels were suppressed, as would be predicted by the above considerations. However, by 18 hours post-sepsis, HMGCR mRNA levels no longer differed from those found in controls. This failure of suppression, at a time of flagrant cholesterol overload, provides strong support for the concepts that dysregulation of the renal HMGCR axis exists in sepsis, and that this potentially contributes to the cholesterol overload state.

To gain further support for the concept of sepsis-induced, HMGCR dependent, cholesterol synthesis, sepsis was simulated in cultured HK-2 cells by the addition of serum harvested from septic animals. Compared to results obtained with control serum, approximately a 40% increase in cellular cholesterol levels resulted. That mevastatin completely abrogated this response proves that the HK-2 cell cholesterol increase was HMGCR-dependent. It is notable that HK-2 cell cholesterol levels remained normal following exposure just to *E. coli* in the presence of normal serum. Hence, sepsis-triggered cytokines, or "stress factors," rather than bacterial products per se seemingly drove the enhanced HK-2 cell cholesterol synthesis. The specific serum factor(s) responsible for this action remain to be defined. Nonetheless, these in vitro data provide critical support for our concept that sepsis-associated cholesterol accumulation occurs, at least in part, by an HMGCR-dependent mechanism. The results obtained with septic serum also provide a completely new insight into mechanisms of stress-mediated renal cholesterol accumulation: they prove that the latter is not strictly a renal injury-based phenomenon, since systemic factors can clearly impact the extent of the renal cholesterol overload state. In this regard, it is noteworthy that a number of cytokines and eicosinoids can impact cellular cholesterol homeostasis, as well as vice versa. This underscores the potential broad reaching implications of the present observations vis-a-vis injury responses at both the epithelial, and possibly the vascular cell, level [37–41].

As previously noted, cellular cholesterol accumulation could occur also via enhanced uptake from plasma cholesterol pools. For example, this might result from LDLR-mediated cholesterol endocytosis [19, 20], or by cholesteryl ester import via plasma membrane-associated SR-B1 [42–44]. The available evidence argues against these two possibilities. First, LDLR knockout mice did not have blunted cholesterol accumulation in response to sepsis, seemingly excluding an LDLR-dependent mechanism. Second, SR-B1 protein expression was clearly decreased, both in post-septic mice, as well as in another model of renal cholesterol overload, that is, the glycerol model of ARF. Thus, increased CE uptake via this pathway seems unlikely. To our knowledge the present results are the first to document reductions in SR-B1 following renal injury. That it occurred in two highly divergent forms of renal injury suggests that SR-B1 suppression could be a previously unrecognized component of the renal stress response.

Scavenger receptor B_1 is thought to serve a dual role in cellular cholesterol homeostasis. As noted above, it may be important in the selective uptake of circulating cholestryl esters. However, it also plays a role in "reverse cholesterol transport," a process that transfers intracellular free cholesterol to HDL particles at the plasma membrane with the latter leading to hepatic clearance [42–45]. Hence, the dramatic decrease in SR-B1 following renal injury has two implications for the present investigations: first, it suggests that increased cholesteryl ester uptake is an unlikely mechanism for cholesterol loading, as discussed above; and second, it provides support for the concept that a decrease in cholesterol efflux may also be operative. ABCA1 transporter is also a cholesterol efflux pathway [21, 42–46]. However, it is minimally expressed in renal tissues, at least as assessed by a lack of its detection by Western blotting (unpublished data; RZ). Given these observations, it seems unlikely that a further-down regulation of the ABCA1 transport mechanism exists. In summary, the available data suggest that sepsis-triggered renal cholesterol loading seemingly involves: (*a*) HMGCR stimulation by circulating cytokines/stress factors; and (*b*) marked reductions in SR-B1, which presumably reduce cholesterol efflux, and hence, magnify the cholesterol overload state.

The final goal of this study was to confirm that sepsis syndrome, like other forms of renal injury, induces a cytoresistant state that is expressed directly at the cellular level. For example, if proximal tubules are harvested from mice or rats with post-glycerol ARF, post-ischemic ARF or with urinary tract obstruction, they manifest increased resistance to in vitro attack [16–18]. The present results indicate that sepsis-mediated renal stress is no exception, given the finding of partial tubular cell resistance to hypoxic and toxic attack. Thus, these findings provide in vitro support for in vivo observations that post-endotoxin-induced renal stress protects against ARF [47]. Choles-

terol's link to the establishment of this cytoresistant state has previously been demonstrated [1, 7, 8]. However, it should not be inferred that cholesterol is the sole factor in initiating cytoresistance. For example, an elegant series of studies by Nath et al have demonstrated that heme oxygenase and ferritin induction also are involved [11, 12, 47]. Finally, it is noteworthy that an increase in both FC and CE were observed in post-sepsis isolated tubules, and not simply in renal cortex. This confirms that the observed renal cortical cholesterol increments reflect, probably in large part, proximal tubular events.

In conclusion, the present studies provide the following new insights into mechanisms of cholesterol accumulation in the setting of experimental sepsis: First, sepsis causes the generation of circulating factor(s), presumably cytokines, which can directly stimulate tubular cell cholesterol synthesis. To our knowledge this indicates for the first time that renal stress-induced cholesterol accumulation is not purely determined by intra-renal events. Second, sepsis causes a marked suppression of SR-B1, a result that would be expected to decrease cellular free cholesterol efflux. In combination, these two mechanisms, plus a failure of HMGCR mRNA suppression, presumably disrupt normal cholesterol homeostasis, and hence, lead to a cellular cholesterol overload state; Third, SR-B1 reductions also occur in the setting of myohemoglobinuric ARF. That two highly divergent models of renal injury (sepsis and rhabdomyolysis) each cause SR-B1 suppression suggests that this new finding may reflect a generic renal response to cellular "stress." Fourth, augmented cellular cholesterol influx does not appear to be a dominant mechanism for sepsis-induced cholesterol accumulation for the following reasons: (*a*) LDLR knockout mice and control mice have comparable FC and CE accumulation following *E. coli* injection; and (*b*) the down-regulation of SR-B1, noted earlier, would be expected to decrease CE influx (while at the same time decreasing FC efflux to circulating HDL). Fifth, the finding that statin therapy dramatically increases hepatic, but not renal cortical, HMGCR mRNA suggests that minimal renal bioavailability may exist (at least to the majority of renal cortex). This raises the possibility that when statins are administered to patients with renal disease, much of their potential beneficial actions may stem from changes in circulating cytokines or lipids, rather than from a direct renal effect. Whether the present results obtained with atorvastatin can be extrapolated to all currently available HMGCR inhibitors, and whether statins might directly impact sub-compartments within the renal cortex (such as the microvasculature or glomeruli) remain to be defined.

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